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14. ABSTRACT

The efforts during this period include 1) the exploration of dendrimers that provide water solubility in the presence of hydrophobic peptides (a limitation encountered in the previous period), 2) the production of dendrimers with a DOTA-chelating ligand that can host the imaging agent, and 3) production of candidate theranostic dendrimer-peptide platforms for the evaluation of biological activity. A new conjugation strategy has been developed and shown to be useful for making the proposed multivalent conjugates. Samples continue to be submitted to our collaborators for biological evaluation.

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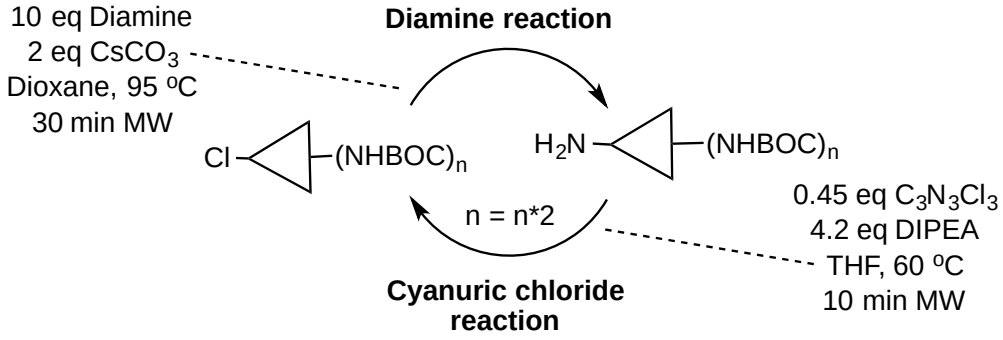
INTRODUCTION: The goals of this multiyear effort focus on the synthesis and characterization of dendrimers that vary in size from generation 3-7 with a functional alkyne core (0-6 mo). This core will be used to install a chelate group for diagnostic medical imaging (4-12 mo). The surface groups will be surveyed for optimal behavior in the therapeutic application (9-18 mo). Therapeutic peptides will be designed and installed (12-24 mo), and the cycle will be iterated over the period of the project with feedback from collaborators at the University of Texas Southwestern Medical Center.

BODY: The organization follows the individual items in the SOW with each constituting an independent, numbered section.

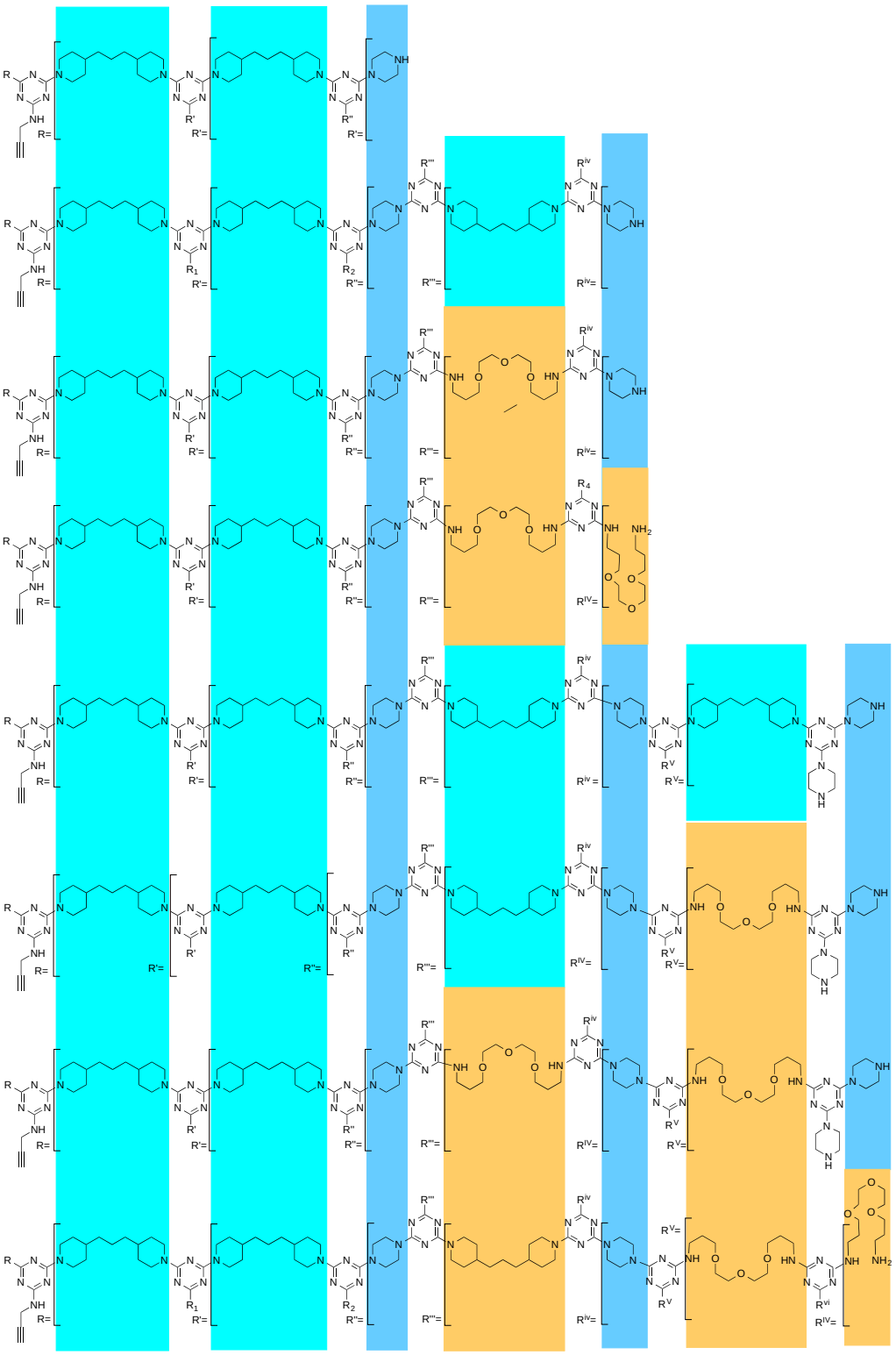
1. Synthesis and characterization of dendrimers that vary in size from generation 3-7 with a functional alkyne (0-6 mo). This work is now complete. Building from efforts that were reported simultaneously with the award of the grant, odd generation dendrimers have been prepared through generation 13. [1] These materials can be prepared to bear an alkyne core. Indeed, to proactively address solubility concerns, two different classes of dendrimers based on their linking chemistry have been examined. These are shown in Chart 1.1. Hydrophobic domains are indicated in blue. Preferred hydrophilic domains are indicated in gold.

Studies during this period also have revealed that rapid, microwave-facilitated syntheses can provide the desired materials to generation 7. [2,3] The scheme is shown below.



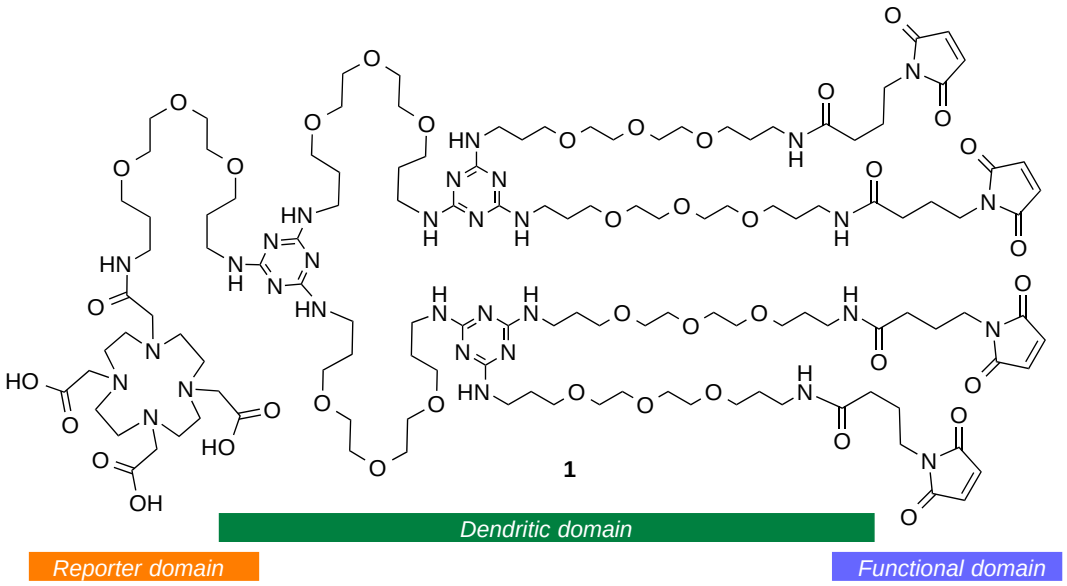
2. Installation of the chelate group for diagnostic medical imaging (4-12 mo). Syntheti routes have been developed that carry a chelating group—DOTA available commercially in large amounts—through the dendrimer synthesis. We now show that tetravalent platforms can be prepared using this strategy (Chart 2.1). Proof-of-principle

Chart 1.1 Hydrophilic domains that promote water solubility appear gold. Hydrophobic domains are blue.



efforts now show that this strategy can be elaborated to larger generation dendrimers. While delaying installation of the diagnostic imaging group cuts down on the amount of the group required to be used due to losses that are intrinsic to any multistep synthesis, the need for advancing biological candidates is critical.

Chart 2.1 A tetravalent scaffold with a DOTA-chelate. The maleimides can be functionalized with amino acids, dipeptides, nonapeptides and oligomers containing 19 amino acids.

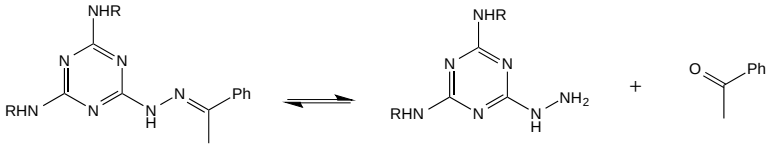


3. Exploration of surface groups to promote desired behavior (9-18 mo). To promote solubility, efforts have instead focused on the dendrimer core groups instead of peripheral groups. During this period, we have developed a useful strategy for imparting greater solubility, and now can balance these solubility needs with the demands of synthesis. In short, synthetically more demanding modules that promote water solubility can be installed on a synthetically accessible platform that suffers from reduced solubility to an end where the final construct has the desired

solubility properties [2]. These results are captured in Chart 1.1. Specifically, cationic groups are not all the same; the hydrophilic linker dramatically improves water solubility over piperazine groups.

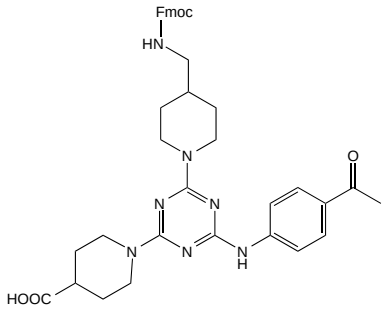
4. Therapeutic peptides will be designed and installed (12-24 mo). These efforts are now the core of our efforts, as translating the original monovalent ligand into a construct with the desired biological activity of the free peptide is of the greatest emphasis. The results of a biolabile linkers study is now being prepared for publication. [4] These are shown in Chart 4.1.

Chart 4.1 A triazinyl hydrazone in equilibrium with the triazinyl hydrazine and free ketone.



In addition, a bifunctional "releasable" agent was also designed and probed for viability. Chart 4.2 This design-element is new to the project. [5] The lack of bioactivity in key constructs from the last period suggested that instead of simply releasing the bioactive peptide, the peptide and a secondary agent (ie. a cationic sequence to promote membrane trafficking) can now be simultaneously released.

Chart 4.2 A releasable agent that can be incorporated into solid phase peptide synthesis.



KEY RESEARCH ACCOMPLISHMENTS:

Dendrimer solubility properties/synthetic burden optimized. Specifically, the dendrimer must bear a significant fraction of lesser reactive, flexible triethyleneglycol containing diamines. Piperazine groups on the surface do not promote solubility when compared to the former diamines.

A route to install the chelate group earlier in the synthesis has been developed using a commercially available agent. The agent hosts gadolinium (and has been shown to host copper). Specifically, the DOTA agent greatly accelerates efforts instead of relying on custom synthesis agents. Such agents can be incorporated when the therapeutic challenges have been met.

Tetravalent architectures have been prepared. Specifically, the tetramaleimides offer opportunities to probe therapeutic potential. Preliminary biological testing of has not reproduced the activity observed in the monomeric ligand upon which the studies were advanced. Indeed, release of the ligand may be critical.

Release kinetics of triazinyl hydrazines have been assessed in model systems and show promise for the proposed activity. Specifically, the perceived critical nature of ligand release can now be assessed.

A bifunctional linker has been prepared that condenses with a suitable linker ketone for conjugation to the dendrimer. Specifically, this group can be incorporated into solid phase peptide synthesis affording a convenient UV-handle and the chance for incorporating two different peptide domains—therapeutic and targeting as originally inspired by the proof-of-concept monomeric ligand.

REPORTABLE OUTCOMES: No reportable outcomes to date.

CONCLUSION: Efforts ongoing are considered to be in-line with milestones identified in the original SOW with key barriers to the identification of a theranostic overcome, and existing challenges well articulated. No deviation from the proposed SOW is requested. The search for bioactive constructs continues.

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[1] Synthesis of Large Dendrimers with the Dimensions of Small Viruses**.** Lim, J.; Kostiainen, M.; Maly, J.; da Costa, C.P.; Annunziata,O.; Pavan, G.M.; Simanek, E.E. *J. Am. Chem. Soc.* **2013**, *135*, 4660-4663.

*[2] Influence of linker groups on the solubility of triazine dendrimers. Enciso, A.E.; Garzoni, M.; Pava, G.M.; Simanek, E.E. *New J. Chem.* **2015**, 39, 1247.

*[3] Microwave-assisted synthesis of triazine dendrimers—a 24 hour challenge. Enciso, A.E.; Simanek, E.E *Manuscript in preparation for Chem. Commun.* Submission date Nov. 2014.

*[4] Triazinylhydrazines: Bifunctional, biolabile linkers for hydrolytic release. Lee, C.S.; Ji, K.; Simanek, E.E. *Outline in preparation for Bioconjugate Chem.* Submission date: Dec 2014.

[5] Hydrolytic release of aldehydes and ketones from a triazinylhydrazines—an acylhydrazine equivalent. Ji, K,; Simanek, E.E. *Manuscript in preparation for Org. Lett.* Submission date: Jan 2015.

*This manuscript cites the project grant.

APPENDICES: Manuscript follows on pg 9.

SUPPORTING DATA: None included.

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PAPER

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Influence of linker groups on the solubility of triazine dendrimers†

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Eight triazine dendrimers were prepared to probe the impact of linker choice on water solubility. Three different linkers were assessed including two hydrophobic diamines that show high reactivity, piperazine and trismethylene bispiperidine, as well as a hydrophilic diamine, 4,7,10-trioxotridecane-1,14-diamine, which is less reactive. Dendrimers **1–8** share a common, generation two, hydrophobic core, **1**. Dendrimer **1** is insoluble in water. Of the three generation four dendrimers, **2–4**, that were prepared, **2** is also insoluble in water, but substitution of one or two of the hydrophobic linkers with 4,7,10-trioxotridecane-1,14-diamine yields sparingly soluble **3** and more soluble **4**, respectively. Molecular dynamics simulations of dendrimers **2–4** in water provide additional insight into their shape, hydration and hydrophobicity. Generation six targets, **5–8**, are also sensitive to choice of interior and surface groups. Dendrimer **5** is insoluble in water, but replacing one or two hydrophobic linkers with 4,7,10-trioxotridecane-1,14-diamine yields dendrimers **6** and **7** with modest affect unless the double substitution occurs in tandem at the periphery to yield **8** which shows high solubility in water. The solubility trends suggest that the choice of cationic surface group is critical, and that piperazine groups on the periphery and interior do little to promote solubility of triazine dendrimers in water compared with the hydrophilic amine 4,7,10-trioxotridecane-1,14-diamine.

Introduction

Dendrimers are often considered for applications that require solubility in aqueous solutions including their use as drugs and drug delivery vehicles.[1] Solubility behavior is most commonly attributed to the nature of the surface groups which can be chosen to manipulate the type and density of charge or other solubilizing groups like poly(ethyleneglycol).[2] In addition to the periphery, the interior groups of triazine dendrimers are subject to facile manipulation because the dendrimers comprise triazine branching points and linking diamines.[3] Historically, the selection criteria for the diamine—while influenced by whether a convergent or divergent approach was being adopted—rested on (i) the reactivity of the diamine, (ii) its cost, and (iii) the commercial availability of protected derivatives. Given the wealth of diamines that meet many of these expectations, the choice of diamine incorporated into triazine dendrimers has evolved over time.

Initially, *p*-aminobenzylamine was favored because the difference in the relative reactivity of the individual amines offered an opportunity to execute a convergent syntheses without functional group interconversions or protecting group manipulations.[4] However, reactivity was sluggish in comparison to other choices like 4-aminomethylpiperidine and intermediates containing the former discolored over time.[5] Piperazine became a linker of choice for small dendrimers due to its high reactivity (compared with primary amines) and the commercial availability of a low cost BOC-derivative.[6] Unfortunately, synthesis was limited to generation 3 targets due to solubility limitations attributed to the disc-like shape of the molecules.[7] To preserve the reactivity of constrained diamines and introduce flexibility, trismethylene bispiperdine was explored. The dendrimers that resulted displayed higher solubility than all-piperazine molecules, but access to high generation materials was still limited based on solubility.[8]

More recently, 4,7,10-trioxotridecane-1,14-diamine has been employed (Chart 1). Here, we probe the role of combinations of these different amines on the solubility of the resulting dendrimers. Chart 2 shows the targets in this phenomenological

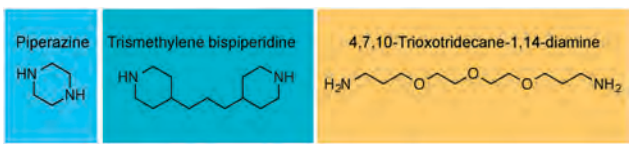

Chart 1 Diamines employed in this study.

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† Electronic supplementary information (ESI) available: Complete experimental procedures, spectral data, and schemes. Figures associated with computation. See DOI: 10.1039/c4nj00917g

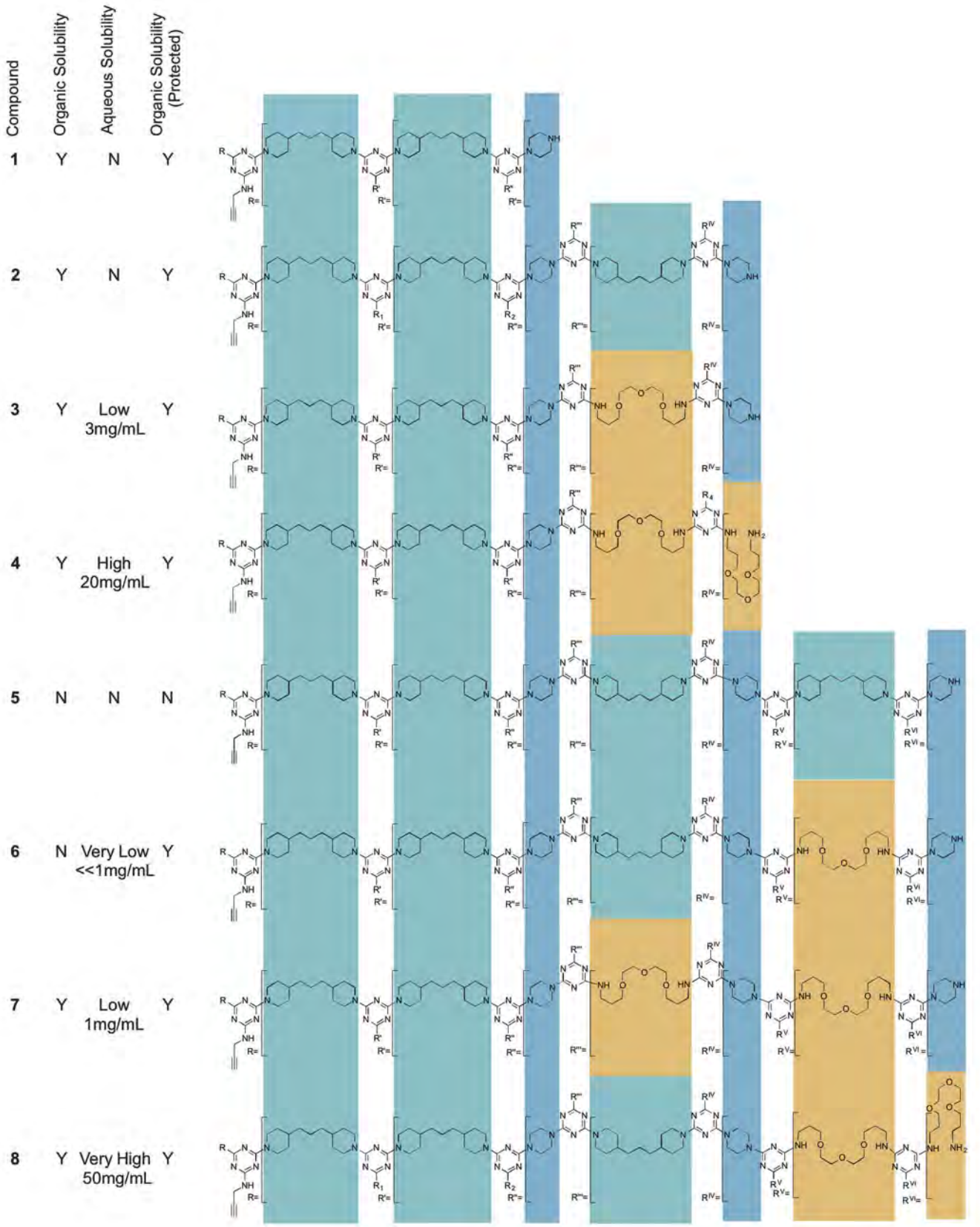

Chart 2 Dendrimers examined in this study. The organic and aqueous solubility data are shown in columns 2 and 3. Column 4 reports the solubility of the BOC-protected precursor in organic solvent.

study. Sharing a common hydrophobic core, triazine dendrimers **1–8** were chosen to anchor our intuition on both (i) the nature of the cationic peripheral group and (ii) the influence of piperazine groups in conveying solubility.

Results and discussion

All targets examined in this study derive from a hydrophobic interior with modest flexibility. This generation 2 dendrimer, **1**, displays 8 piperazine groups on the surface and an alkyne at the core. Dendrimer **1** can be elaborated with macromonomers **9–11** shown in Chart 3 to yield the compounds **2–8**. Specifically, **1** is reacted with **9**, **10**, or **11** to yield BOC-protected derivatives of **2**, **3**, or **4**, respectively. Deprotection with a 1 : 1 mixture of MeOH and conc., HCl yields **2**, **3**, or **4**. Dendrimers **2** and **3** are further reacted with **9**, **10**, or **11** to yield protected **5–8** which are similarly deprotected. Solubility tests were performed by addition of dendrimer to one milliliter of Millipore water (18 MΩ cm). Sonication was used to determine saturation as measured with the naked eye. The solubility observations are reported in Chart 2.

The data anchors our intuition about solubility in three ways. First, substitution of the flexible hydrophilic linker 4,7,10-trioxotridecane-1,14-diamine for hydrophobic trismethylene bispiperidine within the interior of the dendrimer conveys critical, albeit slight water solubility to dendrimers. That is, **2** is insoluble while **3** is sparingly soluble. Similarly, **5** is insoluble while **6** and **7** show increased solubility. Solubility increases from **5** < **6** < **7** as do the number of substitutions of 4,7,10-trioxotridecane-1,14-diamine for trismethylene bispiperidine. Second, the nature of the cationic peripheral group appears to have a profound affect on solubility. That is, replacing the terminal piperazine group with 4,7,10-trioxotridecane-1,14-diamine at the dendrimer surface significantly increases solubility. Dendrimer **4** is much more soluble than either **2** or **3**. Similarly, dendrimer **8** is much more soluble than **5**, **6**, or **7**. Third, the trends are conserved across all generations. Dendrimers **1**, **2** and **5** are all insoluble, and solubility decreases in going from **3** to **6**. This is surprising at some level, as the behavior at the onset of globular structure—predicted here to be at generation 5—might be expected to affect this trend.

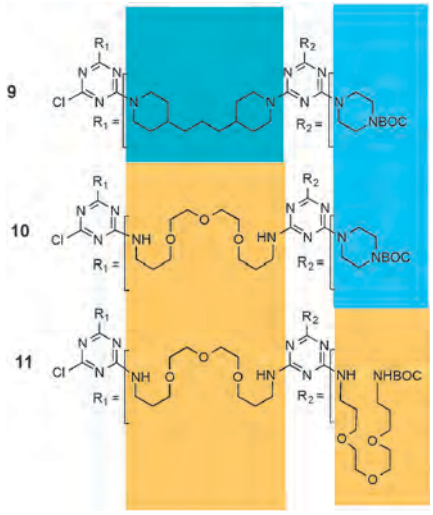

Chart 3 Macromonomers used in the synthesis.

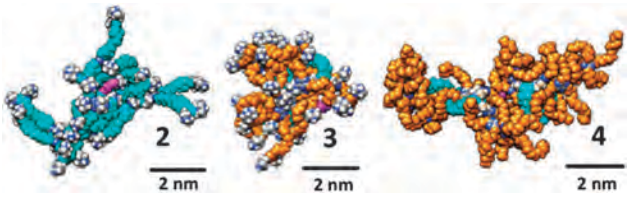

Fig. 1 MD simulation of **2–4** dendrimers in solution. Equilibrated (last) snapshot taken from the MD simulations of **2**, **3** and **4**. Trismethylene bispiperidine and 4,7,10-trioxotridecane-1,14-diamine are colored in cyan and orange respectively. The central residue of the dendrimers is colored in pink. Piperazine and all other groups are colored per-atom (C: grey, N: blue, H: white), water molecules and ions are not shown for clarity.

Computation offers further insight into the role of these linkers in solvation. Molecular models of **2–4** were immersed in a periodic simulation box containing explicit water molecules and 150 mM NaCl and were investigated by means of molecular dynamics (MD) simulations. Equilibrated configurations of **2** and **4** in solution were obtained within 250 ns. Dendrimer **3** required a longer equilibration time, and the MD simulation in this case lasted 400 ns (see ESI†). The equilibrated configurations of **2**, **3** and **4** shown in Fig. 1 reflect the color scheme adopted throughout the manuscript with cyan representing trismethylene bispiperidine and orange representing 4,7,10-trioxotridecane-1,14-diamine.

Computation reveals differences that are consistent with the solubility trend observed. In all cases, the hydrophobic core is sufficiently rigid that complete hydrophobic collapse is precluded. Instead, **2** displays an extended hydrophobic surface that leads us to hypothesize a role in promoting precipitation. For **3** and **4**, the 4,7,10-trioxotridecane-1,14-diamine groups collapse to partially shield hydrophobic domains. This behavior is consistent with previous simulations[9] and typical of the behaviour of PEG chains in water.[10] The shapes of **3** and **4** differ: **3** appears more spherical/globular while **4** adopts an elongated oval shape. Table 1 summarizes computed parameters for **2**, **3** and **4**. At the equilibrium, **3** has smaller radius of gyration (R_g) than **2**. We hypothesize the difference derives from the flexibility differences between 4,7,10-trioxotridecane-1,14-diamine and trismethylene bispiperidine. This difference is reflected in the solvent accessible surface area (SASA): **2** has a

Table 1 Structural features of dendrimers **2–4** obtained from the equilibrated phase MD simulations

Dendrimer	MW (Da)	R_g (Å)	SASA (Å^2)	Density[a] (Da Å^{-3})	H (kcal mol^{-1})
2	10 487	19.2	10 193	0.35	−11 254 ± 26
3	10 646	17.5	9409	0.47	−12 812 ± 35
4	14 940	21.2	13 116	0.37	−15 914 ± 39

[a] Density parameter is calculated by dividing the dendrimer MW for the volume of a sphere with radius R_g.

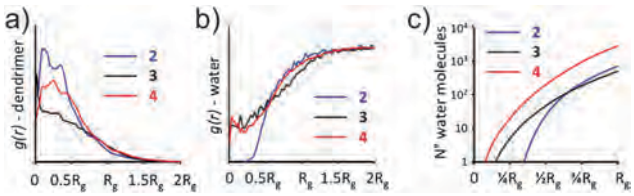

Fig. 2 Radial distribution functions, $g(r)$, obtained from the equilibrated phase MD simulations. The $g(r)$ curves are indicative of the probability to find atoms of the dendrimers (a) and water molecules (b) at given distance from the dendrimer's center of mass (CM). (c) Number of water molecules.

slightly larger SASA than **3** (10 193 A^2 *versus* 9049 A^2, respectively). The SASA of **4** is greater as would be inferred from the depiction, 13 116 A^2 and consistent with the larger R_g of 21.2 Å.

The enthalpy (H) values of the three dendrimers were calculated from the equilibrated phase MD simulations. A relative comparison of H shows that **2** is least stable (relatively) in water. Dendrimers **3** and **4** have more favorable (negative) H values indicating higher relative stability. By comparing calculated H values, we can quantify differences in stability (ΔH) of the dendrimers with respect to **2**. With similar numbers of atoms, **2** and **3** differ in enthalpy with $\Delta H = -1558 \pm 61$ kcal mol^{-1}, indicating that the substitution of trismethylene bispiperidine with 4,7,10-trioxotridecane-1,14-diamine has positive effect on the stability of **3** in water. Consistently, the ΔH of **4** is even more favourable difference with $\Delta H = -4659 \pm 66$ kcal mol^{-1}.

Radial distribution functions $g(r)$ were calculated from the equilibrated phase MD trajectories to probe hydration of **2**, **3** and **4** (Fig. 2). The $g(r)$ curves represent the relative probability to find dendrimer atoms (Fig. 2a) or water molecules (Fig. 2b) at a given distance from the dendrimer's center of mass (CM). Higher $g(r)$ peaks correspond to a high density of atoms and/or restricted migration, while low and broad peaks are indicative of higher molecular flexibility. In all cases, distances from CM are expressed in R_g units to allow comparison between different size dendrimers.

Fig. 2 reveals poorer solvation for **2** compared with **3** and **4**. In fact, the $g(r)$ curves show the dense core of **2** (Fig. 2a, blue) reduces the probability of hydration to 0 at distances lower than $\approx 1/2R_g$ (Fig. 2b). On the other hand, the same results demonstrate that **3** and **4** have a higher levels of core hydration. Fig. 2c plots the number of water molecules present in the interior of the dendrimers. This result corroborates experimental observations of solubility with the solvent penetration of **4** > **3** > **2**.

Conclusions

Solubility remains one of the most significant challenges in the synthesis of triazine dendrimers and limits the generation that these architectures can reach. Here, we show that solubility is impacted substantially by the choice of linking and surface groups across a range of dendrimer sizes. Piperazine, though inexpensive and highly reactive, does little to convey solubility of architectures in water when appearing on either the periphery or interior. This sensitivity to the source of cation is somewhat curious. Flexibility, as offered by trismethylene bispiperidine, improves solubility only slightly while maintaining high reactivity. The emergence of 4,7,10-trioxotridecane-1,14-diamine as a useful building block for dendrimers comes as a surprise: primary amines sacrifice reactivity. Moreover, the introduction of hydrogen bond donors should seemingly promote aggregation, especially in common organic solvents. However, using **11**, the resulting protected dendrimers are soluble in polar organic solvents including chloroform, dichloromethane, DMSO, ethyl acetate, tetrahydrofuran, dioxane, and methanol. These targets are not soluble in ether (which facilitates purification given the solubility of **11** in ether) or hexanes. The deprotected dendrimers containing **11** are soluble in the same subset of organic solvents with the exception of ethyl acetate.

The results obtained here are consistent with our recent success in reaching virus-sized dendrimers of generation 13.[11] There, 4,7,10-trioxotridecane-1,14-diamine was used exclusively as the linking diamine. Surmountable challenges to solubility in water appeared at generation 9, persisted through 11, and were deemed limiting at 13. When 4,7,10-trioxotridecane-1,14-diamine and piperazine linkers are alternated at each generation, dendrimers up to generation 9 were obtained.[12] Generation 11 materials were insoluble in both the protected form (in common organic solvents) and deprotected (in water).

The criteria for diamine choice continue to be refined. While these studies suggest that solubility of an advanced dendrimer might be rescued using macromonomers such as **11**, there appears to be additional room for improvement. Diamines that confer the reactivity of constrained secondary amines like piperazine and trismethylene bispiperidine and retain the advantageous solubility properties of 4,7,10-trioxotridecane-1,14-diamine could prove optimal in our pursuit of the rapid synthesis of large triazine dendrimers. Further experiment and computation will be required to meet these challenges.

Experimental

Molecular dynamics (MD) simulation

The simulation work was conducted using the AMBER 12 software.[13] The molecular models for **2**, **3**, and **4** dendrimers were created and parameterized according to a validated procedure used previously for similar derivatives.[9,14] In particular, **2**, **3**, and **4** dendrimers were parameterized with the "general AMBER force field (GAFF)" (*gaff.dat*).[15] The *parm99* all-atom force field (*leaprc.ff99*)[16] was used to parameterize all the other standard residues present in the simulated molecular systems.

The models of the three dendrimers were placed in a periodic box containing explicit TIP3P water molecules[17] and the necessary number of ions to neutralize the systems and reproduce the experimental ionic strength of 150 mM [NaCl]. Each system underwent initial minimization, and further heating through 50 ps of *NVT* MD simulation to reach the temperature of 300 K. During this second step the solute was maintained as fixed and the solvent was relaxed. Following to this phase, all systems

were equilibrated by running *NPT* MD simulations at the temperature of 300 K and 1 atm of pressure under periodic boundary conditions using a time step of 2 femtoseconds. The Langevin thermostat, and a 8 Å cutoff were used for all equilibration runs. The particle mesh Ewald[18] (PME) approach was adopted to treat the long-range electrostatic effects, and all bonds involving hydrogen atoms were treated by means of the SHAKE algorithm.[19] All MD simulations were carried out using the *pmemd.cuda* module of AMBER 12 working on GTX580 GPU cards. The root mean square deviation (RMSD) and radius of gyration (R_g) data were extracted from the MD trajectories with the *ptraj* module of AMBER 12 and were used to assess the equilibration of each dendrimer (see ESI†). In this respect, while a simulation time of 250 ns was enough for **2** and **4** to reach the equilibrium with good stability, having a sufficiently long equilibrated phase to allow for satisfactory analysis of the MD trajectory (the last 100 ns of MD simulations), a longer simulation time was necessary for **3** (400 ns).

The enthalpy (H) values for **2–4** dendrimers were calculated directly from the equilibrated phase MD trajectories according to the MM-PBSA approach.[20] H is the sum of the total gas-phase *in vacuo* non-bond energy (ΔE_{gas}) of the dendrimers and of a solvation term ($\Delta G_{solv} = \Delta G_{PB} + \Delta G_{NP}$).[21] The polar component of ΔG_{PB} was calculated according to the Poisson–Boltzmann[22] (PB) approach with a numerical solver implemented in the *pbsa* program of AMBER 12.[23] The non-polar contribution to the solvation energy was calculated as $\Delta G_{NP} = \gamma(\text{SASA}) + \beta$, in which $\gamma = 0.00542$ kcal Å^{-2}, $\beta = 0.92$ kcal mol^{-1}, and the solvent-accessible surface area (SASA) was estimated with the MSMS program.[24]

General procedure for deprotection

Compound **1-Boc** (0.417 g, 0.125 mmol) is dissolved in concentrated HCl (3 mL) and methanol (3 mL) and stirred for 15 h at room temperature. After evaporating the reaction mixture under vacuum, the residue is dissolved in dichloromethane, washed with 5 M NaOH (aq), and passed through a phase separator (Whatman). Evaporation of the organic phase yields **1** (0.316 g, quantitative).

General procedure for addition of macromonomer

A solution of **1** (0.160 g, 0.063 mmol), **9** (1.44 g, 1.01 mmol), and diisopropylethylamine (0.19 mL, 1.0 mmol) in 0.6 mL of THF is stirred at 75 °C for 6 days in a pressure relief reaction vial. After evaporation of the reaction mixture, the residue was dissolved in dichloromethane and washed with brine. The organic layer was passed through a phase separator (Whatman), and evaporated under vacuum. The solid was purified by silica gel chromatography (100% dichloromethane to 9 : 1 dichloromethane : methanol). Compound **12** (0.576 g, 67%)—the protected precursor of **5**—was recovered as a white solid.

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Notes and references

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